VARIATION OF HEMOCYANIN CONCENTRATION IN THE BLOOD OF FOUR SPECIES OF HALIOTIS [1]

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Hemocyanin is a copper-containing protein found in solution in the blood of certain arthropods and molluscs. It combines reversibly with oxygen, becoming blue when oxygenated, and is commonly regarded as a respiratory pigment. Hemocyanin has been recognized for about 100 years, and numerous investigations of its chemistry have been carried out (Manwell, 1960; Prosser and Brown, 1961). In a consideration of the function of a body constituent it is important to have available data on the quantity or concentration of the substance in the body. Data on the amount of hemocyanin actually present in the blood are, however, scanty. Redfield (1934) collected, from several sources, data on the oxygen capacity of the blood of several molluscs, and from these calculated values for the copper content of the blood. From the copper content and the known composition of the hemocyanin from these species he calculated the hemocyanin concentration in the blood of four molluscs. The values obtained, expressed as per cent, were: *Octopus vulgaris,* 5.9–9.1; *Loligo pealei,* 7.2–8.8; *Helix pomatia,* 1.5–3.9; and *Busycon canaliculatum,* 3.7–6.6. The greatest range of variation found in any one species was about 2.6-fold. It may be calculated from data on the copper content of the blood of two of these species (Prosser and Brown, 1961) that the hemocyanin content of *Helix pomatia* blood varies from 2.70% to 4.79% and that of *Octopus vulgaris* blood from 9.40% to 11.40%. Stewart *et al.* (1952) mentioned that the range in total protein concentration in the blood of *Cryptochiton stelleri* was from 1% to 3%.

Woods *et al.* (1958) carried out electrophoretic experiments with blood from 6 to 12 individuals belonging to each of 18 species of invertebrates, including *Loligo pealei* and *Ostrea virginica,* and stated (p. 519) that "within a given species differences in sex, size, or stages of the molt cycle produced no significant difference in the electrophoretic patterns."

Horn and Kerr (1963) have presented the most extensive data on the serum protein and copper (and by inference the hemocyanin) concentrations in the blood of *Callinectes sapidus,* the blue crab. They found a 10-fold variation in the total serum protein concentration and an 18-fold range in the serum copper concentration. There was no relationship of either serum protein or copper concentration to the size of the crab, but it was found that female crabs had significantly higher serum protein and serum copper concentrations. No similar data for any molluscs are available.

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In this paper I will document the low average concentrations and the rather unexpected variability in the amount of hemocyanin found in the blood of four species of *Haliotis*, including data from a total of 172 individuals.

Materials and Methods

Collection of animals

Abalones of the species *Haliotis fulgens, H. corrugata, H. rufescens* and *H. cracherodii* were collected at intervals during the period from 1960 to 1962, mostly from the area around La Jolla, California. The depth from which they were taken varied from the intertidal region down to about 60 feet. Most of these animals were bled on the day that they were collected, while some were kept in aquarium tanks provided with running sea water for periods up to nearly two years before being bled; food (*Macrocystis pyrifera*) was provided during this period.

The numbers of animals included in this study were: *H. fulgens,* 107; *H. corrugata,* 26; *H. rufescens,* 32; *H. cracherodii,* 7.

Bleeding of animals

The abalones were removed from the water, quickly shaken to empty water from the branchial cavity, the shell dried with a towel and each animal weighed. The foot was then wiped with a towel, and a short incision, about 2 cm. deep, was made in the midline of the foot. The blood which welled into the cut from the large pedal sinuses was sucked up quickly with a large pipette, centrifuged to remove the few blood cells (the volume of cells was always less than 0.5%), and stored at 0° C. Blood from which the cells have been removed will be called plasma.

Gonad index

The gonad-hepatopancreas was cut at a point midway between the start and the distal end of the horn. The diameter of the whole organ was measured in two directions at right angles to each other, as was also the diameter of the inner hepatopancreas. The gonad index was calculated as follows:

$$\text{Gonad index} = \frac{\text{Avg. diam. of whole organ}}{\text{Avg. diam. of hepatopancreas}}$$

The sex of the animal was determined at the same time.

Total nitrogen and NPN

Total nitrogen was determined on 1-ml. aliquots of the plasma by the micro-Kjeldahl method (A.O.A.C., 1955).

Non-protein nitrogen was determined by the microKjeldahl method on protein-free filtrates prepared by the tungstic acid method of Folin and Wu (1919).

Protein was estimated by multiplying the protein nitrogen concentration by the conventional factor of 6.25. All determinations were done in duplicate.

Absorbance

Spectral absorption curves of the blood showed a broad, rounded maximum at 560 mμ, as has been found for hemocyanins of other species. The absorbance, at 560 mμ, of undiluted and thoroughly aerated plasma was determined as a measure of the hemocyanin concentration. The absorbance at 280 mμ, a measure of the total protein concentration, was determined after dilution of 1 ml. of the plasma to 25 ml. with 0.5 N NaCl solution. Dilution with distilled water caused precipitation of the hemocyanin.

Copper

Aliquots of plasma (usually 1.0 ml.) were digested with 5 ml. of a mixture of 100 ml. of 70% perchloric acid and 400 ml. of concentrated nitric acid. Copper in the digests was determined according to method B given in Diehl and Smith (1958). In this procedure copper is reduced to the cuprous form, extracted as the 2,2′-biquinolate into isoamyl alcohol, and the absorbance of the colored complex measured at 546 mμ. The addition of hydroquinone in a final concentration of 0.5% to prevent fading of the color, as recommended by Riley and Sinhaseni (1958), was found to give good color stability. All determinations were done in duplicate.

Electrophoresis

Horizontal paper electrophoresis was carried out at room temperature. Barbital buffer, pH 8.6, ionic strength 0.05, was used, and the voltage gradient was about 3 volts/cm. Plasma was applied to the strips without prior treatment; 10, 20, or 30 microliters were applied to each 3-cm.-wide strip. After the separation, the strips were stained with bromphenol blue.

Results

Of the various measurements made on the plasma samples, the one for which most data are available is the concentration of total organic nitrogen. This varied

Table 1

Average and range of total nitrogen and of hemocyanin in 4 species of abalones. Nitrogen as mg./100 ml., protein as gm./100 ml.

	H. fulgens	*H. corrugata*	*H. cracherodii*	*H. rufescens*
No. of animals	107	26	7	32
Total N				
Median	129.0	67.4	91.3	92.2
Low	33.0	28.8	60.5	47.1
High	358.0	275.5	342.5	214.5
Hemocyanin N, Median	86.5	23.8	60.1	—
Hemocyanin				
Median	0.54	0.15	0.38	—
Low	0.030	0.0017	0.210	—
High	1.89	1.53	2.03	—
Maximum variation	63-fold	900-fold	10-fold	—

from 33.9 to 358.0 mg./100 ml. for *H. fulgens*. Wide ranges of variation were also found for the other species (Table I). It will be shown first that the hemocyanin concentration is directly related to the total nitrogen concentration. Then the total nitrogen concentration, as a measure of the hemocyanin concentration, will be compared to the environmental and physiological parameters which have been measured. The tabulated raw data have been reported by Pilson (1963).

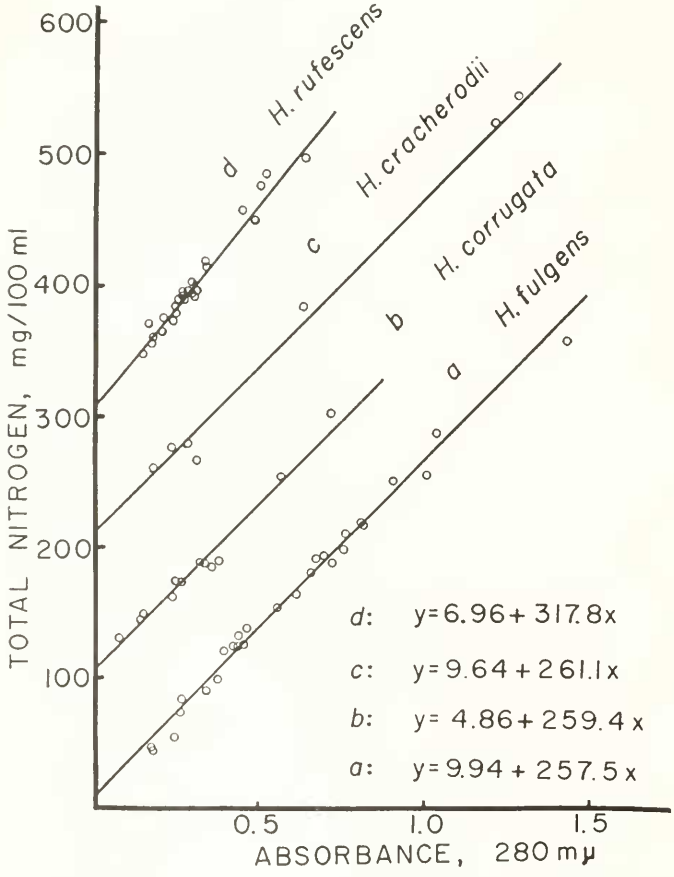

FIGURE 1. Total organic nitrogen concentration in plasma from 4 species of *Haliotis vs.* the absorbance at 280 mμ after dilution of 1 ml. of plasma to 25 ml. with 0.5 N NaCl solution. The plotted points for each succeeding species are displaced upward by 100 units.

Non-protein nitrogen

The median NPN found for *H. fulgens* (14 determinations) was 8.8 mg./100 ml. If the plasma does not contain appreciable quantities of non-protein substances absorbing in the region of 280 mμ, then the NPN can be estimated also from the relation of total nitrogen to the absorbance at 280 mμ. Figure 1(a)

shows this relationship for the 27 samples for which these data were available. This and all subsequent regression lines have been calculated by the method of least squares. The y-intercept at 9.9 is satisfactorily close to the determined median value of 8.8 mg. N/100 ml. There is very little scatter about this line, indicating that large variations in the non-protein nitrogen fraction are unlikely.

Figure 1(b and c) shows the same plots for *H. cracherodii* and for *H. corrugata*. The y-intercept for *H. cracherodii* is 9.6 mg. N/100 ml. and this is the only available estimate for NPN for this species. The y-intercept for *H. corrugata* is 4.9, close to the median value of 5.0 mg. N/100 ml. obtained by direct determination on three samples. Figure 1(d) shows the same relationship for *H. rufescens*, and the estimated average value for the NPN is 7.0 mg. N/100 ml.

These values for NPN are minimal values, for any non-protein substance in the plasma which absorbs at 280 mμ will tend to shift the curve to the right, thus depressing the value at the y-intercept.

The slopes of the regression lines are similar for the first three species, but that for *H. rufescens* is about 23% greater, indicating that the plasma proteins of *H. rufescens* may contain less of the amino acids which absorb at 280 mμ than do those of the other species.

Non-hemocyanin protein

The degree to which the variation in total nitrogen in the plasma is due to non-hemocyanin protein was estimated by paper electrophoresis, the relation of total nitrogen to the absorbance at 560 mμ, and by the relation of the total nitrogen to the copper concentration.

Paper electrophoresis patterns were obtained for samples of plasma from four individuals of *H. fulgens* and one from *H. corrugata*. Two faint bands were present on the strips prepared for all samples, and appeared to be approximately constant in intensity. A third band, of greater mobility than the others, with a mobility approximately that of human α-globulin (human serum was run at the same time as a control) was variable in intensity; by visual estimation this variation in intensity was thought to be approximately proportional to the blueness of the blood and to the total nitrogen content. This band was presumably due to hemocyanin.

All known hemocyanins have, in the oxygenated form, a broad absorption maximum in the region of 560 mμ. Plasma from *H. fulgens* had an absorption spectrum very similar to that of known hemocyanins, with a peak very close to 560 mμ. The absorbance in this region, then, can be taken as a measure of the amount of hemocyanin present. In Figure 2 the total nitrogen in the plasma is plotted against the absorbance at 560 mμ. The intercept of the regression line on the y-axis gives a minimal value for the non-hemocyanin nitrogen present. This is minimal because any other source of absorbance in the plasma will tend to shift the curve to the right, lowering the value for the y-intercept. The value for non-hemocyanin nitrogen is 24.0 mg./100 ml. for *H. fulgens*. The estimated values for the non-hemocyanin nitrogen in plasma from *H. corrugata* and *H. cracherodii* were 21.8 and 20.1 mg./100 ml., respectively. The plot of the values from *H. rufescens*, at the top of Figure 2, shows a different pattern. The 5 points

enclosed in triangles appear to constitute a separate group. It seems that in this species there may be considerable variation in the concentration of the non-hemocyanin protein. No attempt was made to fit a regression line to these points.

Another way to estimate the non-hemocyanin protein is to assume that all the copper in the plasma is bound in the hemocyanin and then to examine the relationship between the total nitrogen present and the amount of copper in the plasma. In Figure 3 the total nitrogen in samples of plasma is plotted against the copper concentration. The regression line fitted to the points from *H. fulgens* has a y-intercept of 42.5. This is probably the best measure of the average amount of

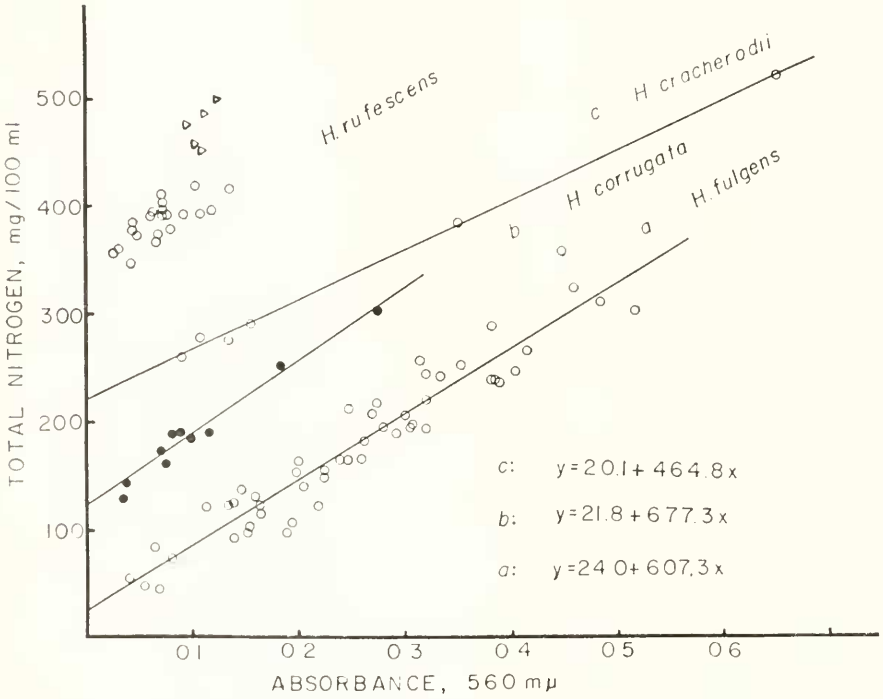

FIGURE 2. Total organic nitrogen concentration in plasma from 4 species of *Haliotis* vs. the absorbance at 560 mμ. The plotted points for each succeeding species are displaced upward by 100 units.

non-hemocyanin nitrogen present. It should be noted, however, that some samples of plasma had less than 42.5 mg. total nitrogen per 100 ml. Similar values for *H. corrugata* and for *H. cracherodii* were 43.6 and 31.2 mg./100 ml., respectively. The plotted data from *H. rufescens* again indicate that in this species the non-hemocyanin protein may be quite variable.

I corrected for the non-protein nitrogen and used the conventional conversion factor of 6.25 to calculate the following average amounts of non-hemocyanin protein present: *H. fulgens*, 204; *H. corrugata*, 242; and *H. cracherodii*, 135 mg./100 ml.

Hemocyanin concentration

Figure 4 shows the relationship between the absorbance at 560 mμ and the copper concentration of the plasma from *H. fulgens, H. corrugata* and *H. rufescens.* It is clear that, for the first two species named above, the absorbance at 560 mμ is well correlated with the copper concentration. This indicates that most of the copper is present in combination as hemocyanin. For both species the y-intercept

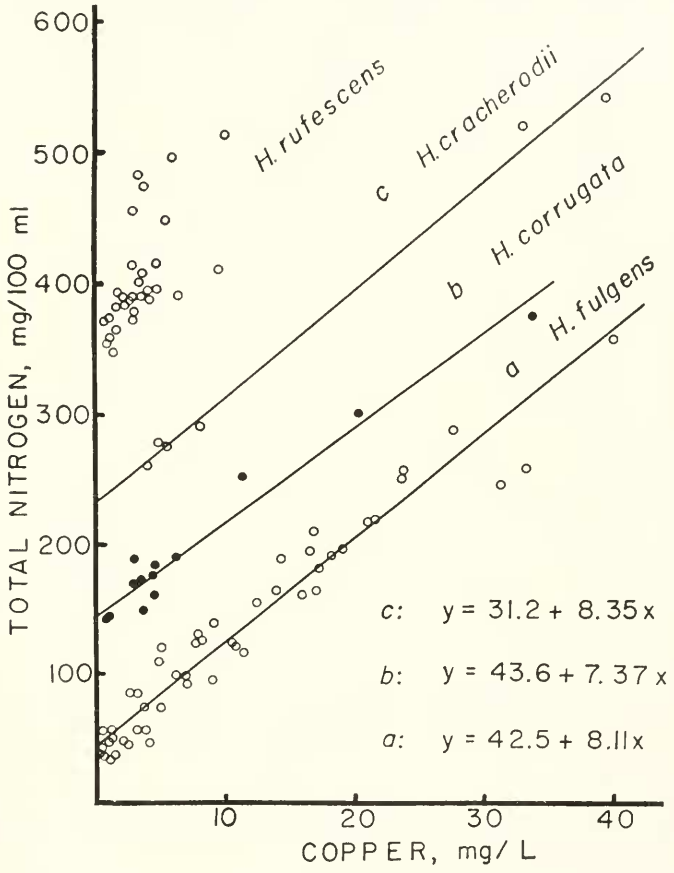

FIGURE 3. Total organic nitrogen concentration in plasma from 4 species of *Haliotis vs.* the total copper concentration. The plotted points for each succeeding species have been displaced upward by 100 units.

is above 0, indicating that the plasmas absorbed or scattered light by an amount greater than could be attributed to the hemocyanin alone.

From the relationships that have been developed it seems that one can with some confidence use the total nitrogen, the absorbance at 560 mμ or at 280 mμ, or the copper concentration, to estimate the hemocyanin concentration in the plasma of all species investigated except *H. rufescens.*

The data which have been presented show that the concentration of hemocyanin in abalone blood is extremely variable. A frequency histogram, at 20-mg. intervals, of the total nitrogen in all 107 samples of plasma from *H. fulgens* is shown in Figure 5. Since the relationship shown in Figure 3 indicated that the non-hemocyanin nitrogen averaged about 40 mg./100 ml., the scale was shifted two intervals (40 mg.) to show an approximation to the frequency histogram for hemocyanin nitrogen. This distribution is not normal. It is relatively flat, and skewed to the right. The usual statistical measures are not entirely appropriate

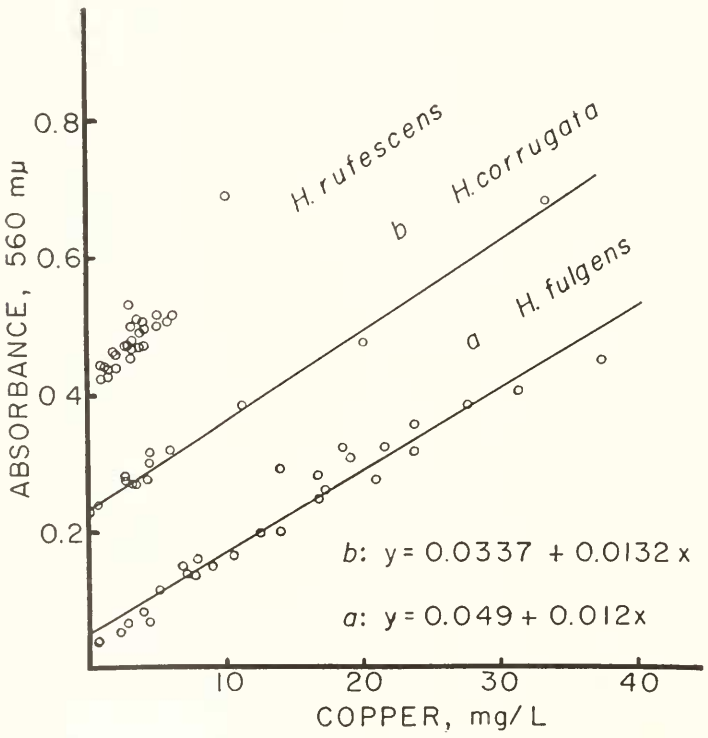

FIGURE 4. Absorbance at 560 mμ of plasma from 3 species of *Haliotis vs.* the total copper concentration. The points for each succeeding species have been displaced upward by 100 units.

to this distribution; I have used the median and the range because I want to emphasize the physiological implications of the great range found.

Though Horn and Kerr (1963) did not point this out, it may be noted from their data that the statistical distribution of serum protein concentrations was different for male crabs, being more skewed toward the high end of the range, and having a much flatter distribution curve in comparison with the more nearly normal distribution of this value for female crabs.

The median value of total nitrogen for these 107 animals is 129.0 mg./100 ml., and the range is from 33.9 to 358.0 mg./100 ml. Subtracting the estimated value of 42.5 mg. of non-hemocyanin nitrogen gives a median value of 86.5 mg. of

hemocyanin nitrogen per 100 ml. This may be multiplied by the factor 6.25 to give a median hemocyanin concentration of 0.54 gm./100 ml.

The range in hemocyanin concentration may be estimated from the total nitrogen figures by subtracting the estimate for non-hemocyanin nitrogen. This gives a range of 0 to 1.97 gm. of protein per 100 ml. However, no samples of plasma were encountered in which copper could not be detected; therefore, the *range* of hemocyanin concentration has been estimated from the copper concentrations. This varies from 0.59 to 37.2 mg. Cu/L. From the relationship given in Figure 3(a) it follows that these copper values correspond to a range of 4.78 to 302 mg. of hemocyanin nitrogen per 100 ml. The corresponding range for hemocyanin concentration is from 0.030 to 1.89 gm./100 ml. There is, then, in

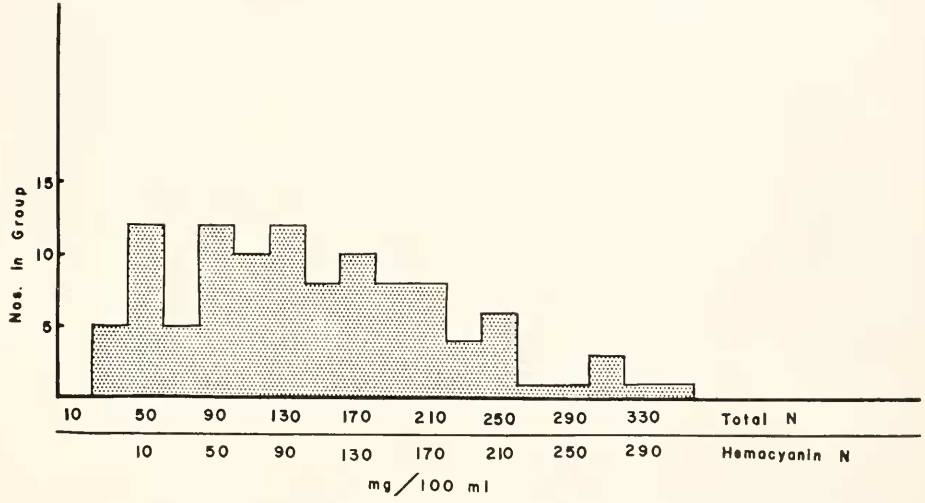

FIGURE 5. Frequency histogram of total organic nitrogen concentration in plasma from *H. fulgens*. The offset scale gives an approximation to the frequency histogram of hemocyanin nitrogen concentration.

these 107 samples of plasma from *H. fulgens*, a 63-fold variation in the concentration of hemocyanin present.

These and similar calculations for the other species are summarized in Table 1. No attempt was made to calculate average hemocyanin values for *H. rufescens*.

Median hemocyanin concentrations were 0.15 and 0.38 gm./100 ml. for *H. corrugata* and *H. cracherodii*, respectively, and the maximum variation found was 90-fold and 10-fold, respectively.

Weight and total nitrogen

Figure 6 shows the concentration of total plasma nitrogen in *H. fulgens* plotted against the weight of the animal. It is clear that the total plasma nitrogen concentration (or, by inference, hemocyanin concentration) is not related to the size of the animal. Probably it is not related to the age of the animal either.

Similar graphs for the other three species are equally unrewarding, and are not illustrated here.

Gonad index and total nitrogen

It might reasonably be supposed that the reproductive activities of the abalone, involving the synthesis of large amounts of gonad tissue and of eggs or sperm, might affect the hemocyanin or total nitrogen concentration of the plasma. Figure 7 shows the total nitrogen concentration in the plasma from *H. fulgens* plotted against the gonad index. It would appear that the total nitrogen of the plasma is unrelated to the reproductive state of the animal, as judged from the gonad index. Similar graphs for the other species show an equal lack of relationship.

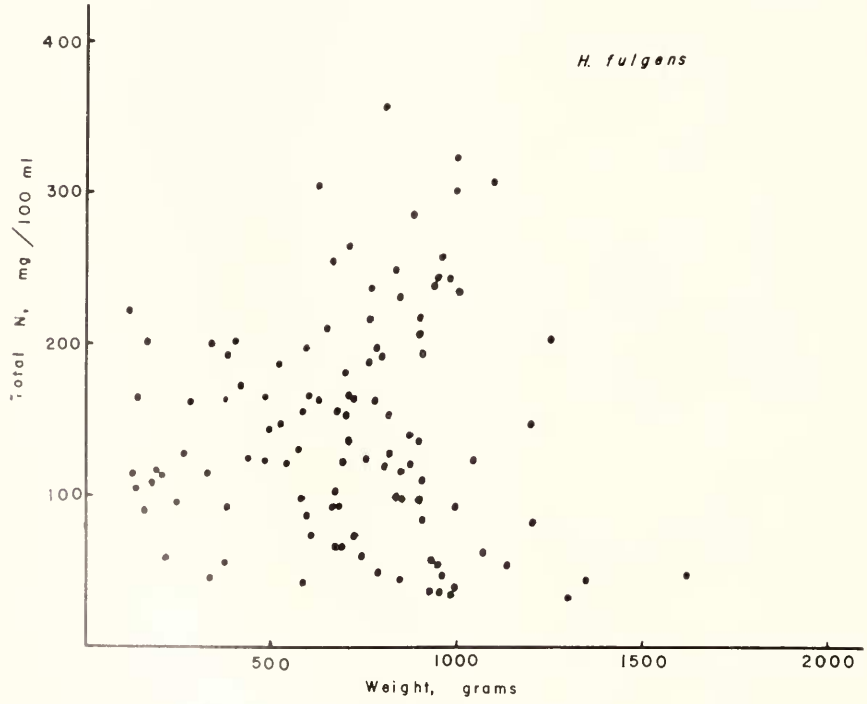

FIGURE 6. Total organic nitrogen concentration in plasma from *H. fulgens vs.* the total weight of the animal.

There was no significant difference in the total nitrogen concentration of the plasma between male and female specimens of *H. fulgens*.

Nutritional state and total nitrogen

Five individuals of the species *H. corrugata* were collected from a depth of 35 feet off Point Loma in an area from which the kelp had been absent for at least a year. These animals all appeared shrunken and the foot muscle was weak and

flabby; the gonads were undeveloped. Another group of five individuals, collected also from a depth of 35 feet only 200 yards away, had been living in an area where kelp was abundantly available. These were firm, healthy in appearance and with well developed gonads. The average total nitrogen content of the plasma from the starved animals was 62.1 mg./100 ml., and that from the well fed animals was 47.5 mg./100 ml. This difference was not statistically significant. From this admittedly small sample, it appears that hemocyanin concentration is not correlated with nutritional state. The great majority, and perhaps all, of the other animals examined were healthy and firm in appearance.

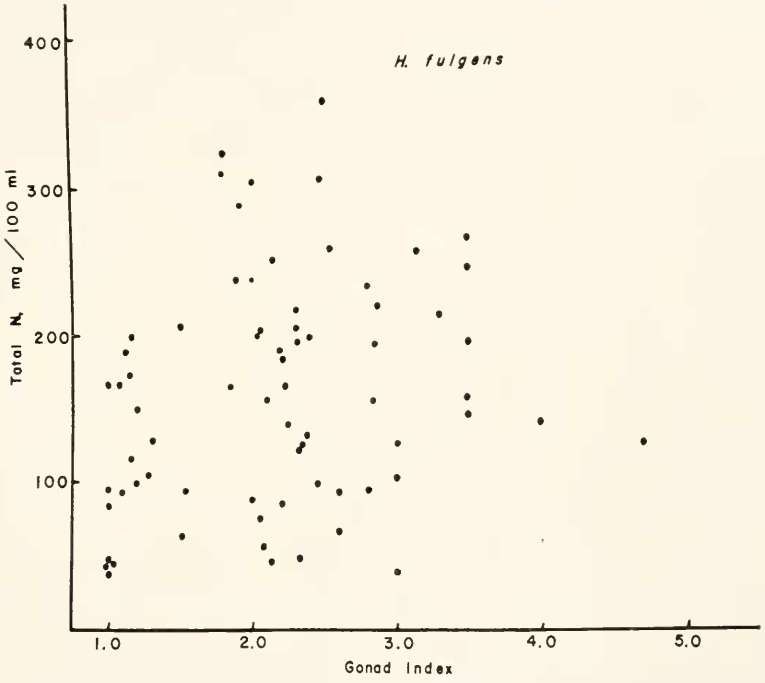

FIGURE 7. Total organic nitrogen concentration in plasma from *H. fulgens vs.* the gonad index.

Depth and season

Within the species *H. fulgens* there appeared to be no relationship between the concentration of total nitrogen in the plasma and the depth at which the animals had been collected.

Thinking that the warming of the water during the summer months might put sufficient stress on the abalones to cause an increased synthesis of hemocyanin, I plotted in Figure 8 the mean total nitrogen in various collections against the time of year. There is no obvious seasonal trend demonstrated in this figure.

Copper and hemocyanin

If it is assumed that hemocyanin from *H. fulgens* contains two atoms of copper for each molecule of oxygen bound, as is known to be the case for other

species, it is possible to calculate, from the relationship shown by Figure 3, the minimum combining weight of the hemocyanin. For *H. fulgens* this is 10,300 gm. of nitrogen. Multiplying by the conventional factor of 6.25 yields a minimum weight of 64,400 gm. of protein. Comparable figures obtained by direct analysis of the hemocyanin from other molluscs are 52,800 for *Helix pomatia*, 51,800 for *Busycon canaliculatum*, and 50,800 for *Octopus vulgaris* (calculated from data in Prosser and Brown, 1961).

Severy (1923) reported the copper content of each of five whole individuals of *H. cracherodii* to be 0.8 mg./kg. of wet tissue. Marks (1938) reported the following whole-body copper concentrations in three species of abalone: *H. fulgens*, 2.5 to 10 mg./kg. (11 samples); *H. cracherodii*, 1 to 13 mg./kg. (6 samples);

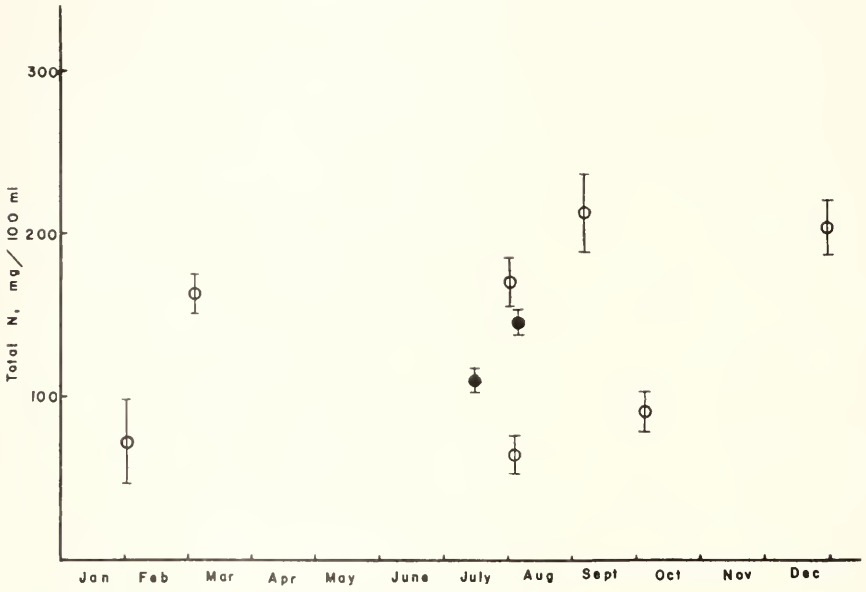

FIGURE 8. Average total organic nitrogen concentration in various groups of *H. fulgens vs.* the time of year when the group was collected. Open circles identify groups bled shortly after collection; closed circles identify groups maintained and fed in aquaria for 7 to 24 months. The limits given are the standard errors of the means.

H. rufescens, 1 to 2 mg./kg. (3 samples). The average blood volume, determined by dilution of inulin, for three individuals of *H. fulgens* was 41% (Pilson, 1963). Thus, in this species the blood alone would contribute from 0.24 to 15.3 mg. of Cu per kg. of wet tissue. The data reported by Marks are more or less in accord with those reported herein, and indicate that, at least in the case of those animals with high concentrations of hemocyanin in the blood, the major part of the total body store of copper is probably present in the circulating hemocyanin.

DISCUSSION

The significance of this great variation in the concentration of hemocyanin in the blood of *Haliotis* to the physiology of the animal is not known. It is a

common view that hemocyanin acts as a respiratory oxygen-carrier, and the known chemistry of this protein is not in conflict with this idea. Certainly hemocyanin in all known species can combine reversibly with oxygen. However, if some animals can survive with a certain concentration of hemocyanin in their blood why should other individuals of the same species have a concentration 60 times greater? It seems that such an enormous variation in the concentrations present is not compatible with any physiological function which has been suggested so far.

That hemocyanin in *Haliotis* might be a sort of evolutionary relic, in the process of disappearing, seems unlikely, for the variability was found in all four species investigated, although their ecologic distributions are different (Cox, 1962), and a range of hemocyanin concentration almost as great was also found in the blue crab (Horn and Kerr, 1963).

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Summary

1. The concentration of non-protein nitrogen in plasma from four species of *Haliotis* was fairly constant with average values estimated as follows: *H. fulgens*, 9.9; *H. cracherodii*, 9.6; *H. corrugata*, 4.9 and *H. rufescens*, 7.0 mg./100 ml.

2. The concentration of non-hemocyanin protein in plasma was fairly constant, and quite low, in three of the species studied. Average values, expressed as gm./100 ml., were: *H. fulgens*, 0.20; *H. cracherodii*, 0.14; *H. corrugata*, 0.24. Plasma from *H. rufescens* contained low but variable quantities of non-hemocyanin protein.

3. Hemocyanin was present in abalone plasma in greatly varying concentrations. The median hemocyanin concentration in the plasma of 107 individuals of the species *H. fulgens* was 0.54 gm./100 ml., and the range was 0.03 to 1.89 gm./100 ml. This is a 63-fold variation in concentration.

4. Plasma from *H. corrugata*, *H. cracherodii* and *H. rufescens* also had varying concentrations of hemocyanin. Plasma from 26 individuals of *H. corrugata* had a median concentration of 0.15 gm./100 ml., and seven individuals of *H. cracherodii* had a median hemocyanin concentration of 0.38 gm./100 ml. The corresponding ranges were 0.0017 to 1.53, and 0.210 to 2.03 gm./100 ml., respectively, giving a 900-fold and a 10-fold range between the highest and lowest samples. No similar information is available for other species of molluscs.

5. The concentration of hemocyanin was unrelated to the animal's weight, sex, reproductive activity, as judged by the gonad index, nutritional state, the depth in the water at which the animal had been collected, or the season of the year.

6. This enormous range in concentration of hemocyanin in the blood appears to be incompatible with any physiological function which has so far been suggested.

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